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# Extracellular Neuraminidase Production by *Pasteurella* Species Isolated from Infected Animals

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**Abstract.** A total of 721 field isolates of various *Pasteurella* species (*haemolytica*, *multocida*, and *testudinis*) from various regions of the United States were examined for extracellular neuraminidase production. All strains were grown and tested in the same way. Included were 372 *P. haemolytica* serotype 1 isolates, 181 *P. haemolytica* serotype 2 isolates, 63 *P. haemolytica* serotype 6 isolates, 101 *Pasteurella multocida* isolates, and 4 *Pasteurella testudinis* isolates. All *Pasteurella* species examined produced the enzyme. The data revealed the following: (1) Several transfers of *P. haemolytica* strains on blood agar medium did not cause a decrease in enzyme activity. (2) *P. haemolytica* serotypes 2 and 6 produce more neuraminidase than *P. haemolytica* serotype 1, *P. multocida*, and *P. testudinis* isolates. (3) There was no apparent change in neuraminidase production by *P. haemolytica* serotypes 1 and 2 obtained from the same animal taken on different days in the feedyard. (4) There was no significant change in neuraminidase production by *P. haemolytica* serotype 2 isolates taken from the same animal at the auction market and later at the feedyard.

Previous studies in our laboratory have examined the production of neuraminidase by various species of *Pasteurella*. This is because of our belief that this enzyme may play a role in the disease process. In 1970, Scharmann and associates [9] reported that three out of five isolates of *P. haemolytica* they examined produced a neuraminidase, as did 102 of 104 *P. multocida* isolates. It was later shown that neuraminidase production by other bacteria as well as protozoa played a role in their ability to produce disease [8]. Gottschalk [5] proposed a possible mechanism for neuraminidase activity in bacterial infections. He demonstrated the protective capabilities of salivary glycoproteins against potentially pathogenic organisms were inhibited by the removal of sialic acid by neuraminidase. It was theorized that in this situation exogenous bacterial enzyme would aid the organism's survival against defense mechanisms in vivo. In 1981, *P. haemolytica* serotypes 1 through 12 were examined for neuraminidase production by Frank and Tabatabai [2]. These authors demonstrated that all serotypes (except 2, 3, 8, 10, and 11) produced this enzyme. It was later shown by Straus and colleagues [11] that all serotypes of *P. haemolytica* (except 11) produced neuraminidase. We have demonstrated that the neuraminidase of *P. haemolytica* serotype A1 (Ph A1 is a heat-labile, extracellular enzyme that is produced maximally in the stationary phase of growth and has a molecular weight of 160,000 [12]. We recently showed that this enzyme is produced in vivo in goats when they are infected with Ph A1 by transthoracic challenge [10]. We have shown that the Ph A1 neuraminidase is similar to the neuraminidases produced by the other *P. haemolytica* serotypes regarding antigenic identity, substrate specificity, and molecular weight [11]. We have recently characterized an extracellular neuraminidase produced by a strain of *P. multocida* A:3 that was isolated from a case of bovine pneumonia [14]. In that study, we demonstrated that the neuraminidases from *P. multocida* A:3 and *P. haemolytica* A1 were not antigenically related. Recently, Ifeanyi and Bailie [6] partially purified a neuraminidase from *P. multocida* A:3 and demonstrated that anti-neuraminidase antibodies pro-

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tected mice against *P. multocida* A:3 lethal infection. The present study was undertaken to examine the association of neuraminidase produced by *Pasteurella* species from infected animals in a representative sampling of the United States. In light of the role that neuraminidase plays in the pathogenesis of other bacteria [8], we sought to determine if there was an elevated production of this enzyme by any serotype of *P. haemolytica*. Serotype A1 of *P. haemolytica* is the one most commonly isolated from pneumonic infections in cattle after their shipment to market [7]. We also wanted to determine if neuraminidase production by *P. haemolytica* changes over time as infected animals traveled through the market system. Finally, we wanted to see if any species of *Pasteurella* produced more extracellular neuraminidase than any of the others.

## Materials and Methods

**Bacterial strains and their isolation.** Seven hundred and twenty one *Pasteurella* species isolates were obtained from various states in the United States. States from which isolates were obtained include Arizona, California, Colorado, Illinois, Iowa, Michigan, Nebraska, Oklahoma, Texas, Washington, and Wyoming. Of the 721 strains of *Pasteurella* species tested, 372 were Ph A1, 181 were *P. haemolytica* serotype 2 (Ph 2), 63 were *P. haemolytica* serotype 6 (Ph 6), 101 were *P. multocida*, and 4 were *P. testudinis*. The *P. haemolytica* strains and the *P. multocida* strains were obtained from nasal swabs from infected cattle and goats, and from the lungs of infected goats and cattle at necropsy which died from their infections. The *P. testudinis* strains were isolated upon necropsy from tortoises which died from their infections. All infected animals were considered to be sick from their *Pasteurella* infection by diagnosis of an independent veterinary diagnostic laboratory.

**Medium, growth conditions, and enzyme assays.** The storage of bacterial strains, the culture medium used, growth conditions, and preparation of culture filtrates (stage I) were performed as previously described [12, 14]. Briefly, *Pasteurella* colonies from blood agar plates were transferred to 10 ml of a chemically defined medium [RPMI-1640 buffered with 25 mM HEPES (pH 7.2) GIBCO, Grand Island, N.Y.]. The tubes were incubated at 37°C for 24 h with shaking (180 rpm). Growth was measured turbidimetrically at 540 nm in a Spectronic 20 colorimeter (Bausch and Lomb, Inc., Rochester, N.Y.). When cultures were ready for harvest ($OD_{540} = 1.0$) they were immediately chilled, and cells were removed by centrifugation (17,700 *g*, 4°C, 45 min) in a J2-21 refrigerated centrifuge (Beckman Instruments, Inc., Fullerton, Calif.). Filtrates were then concentrated to dryness by lyophilization, and the powder was dissolved in 1 ml of 10 mM sodium acetate buffer, pH 6.5 (for *P. haemolytica*) or pH 6.0 (for *P. multocida* and *P. testudinis*) and dialyzed against 2 L (2×) of the same buffer for 48 h.

**Data analysis.** Data were analyzed by a computer program employing the unpaired t-test for enzyme levels [3]. The results were expressed as the mean of duplicate determinations ± SEM.

Table 1. Comparison of in vitro neuraminidase activity by various species of *Pasteurella*

| Groups compared | Number of strains tested | Mean ± standard error[a] | P-value[b] |
|---|---|---|---|
| Ph A1/Ph 2 | 372/181 | 0.78 ± .04/1.47 ± .07 | <0.0001* |
| Ph A1/Ph 6 | 372/63 | 0.78 ± .04/1.29 ± .13 | <0.0001* |
| Ph 2/Ph 6 | 181/63 | 1.47 ± .07/1.29 ± .13 | <0.1886 |
| Ph A1/*P. multocida* | 372/101 | 0.78 ± .04/0.72 ± .08 | <0.5079 |
| Ph 2/*P. multocida* | 181/101 | 1.47 ± .07/0.72 ± .08 | <0.0001* |
| Ph 6/*P. multocida* | 63/101 | 1.29 ± .13/0.72 ± .08 | <0.0001* |
| Ph A1/*P. testudinis* | 372/4 | 0.78 ± .04/0.40 ± .08 | <0.3359 |
| Ph 2/*P. testudinis* | 181/4 | 1.47 ± .07/0.40 ± .08 | <0.0202* |
| Ph 6/*P. testudinis* | 63/4 | 1.29 ± .13/0.40 ± .08 | <0.0902 |
| *P. multocida*/*P. testudinis* | 101/4 | 0.72 ± .08/0.40 ± .08 | <0.4308 |

[a] Cells were grown to the stationary phase in 10 ml of RPMI-1640 plus 25 mM HEPES. Enzyme preparations (stage 1) were prepared as described in the text. Total activity is expressed as nanomoles of sialic acid released per minute per mg protein. The results are expressed as the mean of duplicate determinations ± SEM with fetuin as the substrate.

[b] P-values were determined by the unpaired t-test for enzyme levels. P-values less than or equal to 0.05 indicated that the two groups were significantly different, as indicated by asterisks (*).

## Results

**In vitro neuraminidase production by various *Pasteurella* isolates.** Table 1 shows a comparison of in vitro neuraminidase activity by various species and serotypes of *Pasteurella*. From this table it is clear that Ph 2 and Ph 6 both produce significantly ($P \leq 0.05$) more neuraminidase than does Ph A1. Of the three *P. haemolytica* serotypes we examined, Ph 2 produced more enzyme than did the other two *P. haemolytica* serotypes. Ph 2 produced significantly ($P \leq 0.05$) more neuraminidase than did *P. multocida* and *P. testudinis*. There was not a significant ($P \leq 0.05$) difference in the production of enzyme by *P. testudinis* and *P. multocida*. Other observations regarding neuraminidase production by *P. haemolytica* were made. It was determined that transfer of Ph A1 several times on blood agar over a period of a week did not result in a significant decrease in enzyme activity (data not shown).

**Examination of *Pasteurella haemolytica* neuraminidase production over time.** Isolates were obtained from a number of cattle at auction market and during their stay at the feedyard. We were able to obtain a variety of *P. haemolytica* isolates from these animals and test them for neuraminidase production. There was no change in neuraminidase production by any of

Table 2. Comparison of in vitro neuraminidase activity by *P. haemolytica* serotype 2[a] isolates from the same cow on three different days in the feedyard

| Groups compared | Number of strains tested | Mean ± standard error[b] | P-value[c] |
|---|---|---|---|
| Day FY 8/Day FY 15 | 6/5 | 1.83 ± .26/1.53 ± .35 | <0.5083 |
| Day FY 15/Day FY 29 | 5/6 | 1.53 ± .35/1.47 ± .73 | <0.9387 |
| Day FY 29/Day FY 8 | 6/6 | 1.47 ± .73/1.83 ± .26 | <0.6290 |

[a] Isolates were always obtained from the nostrils of this animal. Isolates were always taken from the same site, were always *P. haemolytica* serotype 2, always produced the same amount of in vitro neuraminidase, and appeared morphologically identical.

[b] Cells were grown to the stationary phase in 10 ml of RPMI-1640 plus 25 mM HEPES. Enzyme preparations (stage 1) were prepared as described in the text. Total activity is expressed as nanomoles of sialic acid released per minute per mg protein. The results are expressed as the mean of duplicate determinations ± SEM with fetuin as the substrate.

[c] P-values were determined by the unpaired t-test for enzyme levels. P-values less than or equal to 0.05 indicated that the two groups were significantly different.

the *P. haemolytica* isolates with time. Table 2 shows the in vitro neuraminidase production by 17 Ph 2 isolates taken from the same animal. Five isolates were obtained after 8 days in the feedyard (FY 8), six isolates were taken from the animal on the 15th day in the feedyard (FY 15), and six isolates were obtained from the animal on its 29th day in the feedyard (FY 29). All 17 isolates were Ph 2. There was no significant difference in in vitro enzyme production among the isolates obtained on the different days.

## Discussion

These studies demonstrate that there is a great deal of variation in in vitro neuraminidase production between various species of *Pasteurella* and even among the various serotypes of *P. haemolytica* (Table 1). Certain serotypes of *P. haemolytica* have been isolated more frequently from cattle with pneumonia than have others. Serotypes 1 and 2 are the main serotypes isolated from cattle nares [1]. However, Ph A1 is the serotype most frequently recovered from cases of acute fibrinohemorrhagic lung infections that develop in cattle after shipping [7]. A study by Tabatabai and Frank [13] examined the relationship between serotype and neuraminidase production by ovine and bovine field isolates. These workers suggested that neuraminidase production by *P. haemolytica* was serotype specific. Our data, examining a much larger number of *P. haemolytica* strains, appear to confirm that finding. Frank and Tabatabai [2] found that serotype 1 *P. haemolytica* isolates produced higher levels of neuraminidase than did serotype 2 strains. However, our data demonstrate that *P. haemolytica* serotype 2 isolates produce significantly higher levels of extracellular neuraminidase than do Ph A1 strains. This can probably be explained by the fact that Frank and Tabatabai examined a small number of strains (approximately 20) and quantitated only cell-bound enzyme activity [2]. In our study, we examined 553 serotype 1 and 2 strains and quantitated extracellular enzyme production. The fact that serotype 2 *P. haemolytica* strains produce significantly more neuraminidase than Ph A1 strains may indicate that the enzyme is not involved in pneumonic pasteurellosis. However, because all the strains examined were isolated from sick animals, a more likely explanation is that neuraminidase production is required for *P. haemolytica* to cause pneumonic infections in cattle, regardless of the serotype. Finally, we showed that repeated transfer (7 days) of *P. haemolytica* A1 did not cause a reduction in the ability of the organism to produce neuraminidase in vitro. The same phenomenon has also been observed for the leukotoxin of Ph A1 [4].

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